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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Telomeres are specialized chromatin structures that protect chromosomes ends from the DNA repair pathways. Telomeres are re-formed after each round of DNA replication. The molecular details of telomere maturation have been well characterized. Many of the details of this process resemble the repair of double strand breaks (DSBs) by the homologous recombination pathway. For this reason, it has been hard to discover what distinguishes telomeres from double stand break lesions. The first step in telomere maturation is the resection of the blunt double stranded end to form a 3' overhang, called a G-tail. This is a critical part of telomere maturation, as it prevents repair of telomeres through non-homologous end joining. Experiments described here have led to the identification an exonuclease, Rat1p, which is required for G-tail formation. Impairing Rat1p activity in dividing cells causes telomeres to become fused through the non-homologous end joining pathway. The identification of Rat1p was unexpected as this protein has been previously characterized as an exoribonuclease with roles in RNA processing and degradation. I have discovered that Rat1p is also able to degrade DNA substrates in the direction required for G-tail formation. In addition, Rat1p was found to associate with telomere chromatin during late S-phase.

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Introduction

It has long been known that an essential role of telomeres is to prevent the recognition of chromosome ends by DNA repair pathways. Repair of telomeres as DSBs can lead to dicentric chromosomes, which are very unstable in dividing cells. A simplistic model to explain this function of telomeres would assume that telomeres prevent DNA repair enzymes access to chromosome ends, thereby protecting chromosomes from accidental fusions. However, the reality is much more complex, and many components of DSB repair pathways participate in normal telomere functions. In fact, it appears as though telomeres and DSBs are much more similar than originally thought. Native telomeres are created when the DNA replication machinery synthesizes telomeres late in S-phase. In order to become functional, a native telomere must then be processed by an as-yet-identified pathway into its mature form. Some of the latest reports in the field provide evidence for a model in which telomeres are first recognized as DSBs, but then are later repaired along a specialized pathway resulting in a mature functional telomere. I propose that much of what we know about DSB repair can be extrapolated onto a model for telomere processing. According to my model for telomere processing (Figure 1), the resection of the C-strand to create a G-tail is what determines whether telomeres are repaired by the NHEJ pathway, or by a more appropriate telomere-specific pathway. If telomeres are repaired by NHEJ, dicentric chromosomes are created, which lead to breakage-fusion-bridge cycles and eventual cell death. It is, therefore, of great interest to the field to identify the enzyme responsible for the resection of telomeres. Based on evidence listed below, I propose that this exonuclease is Rat1p.

Body

1. **Experiments that test whether Rat1p has a direct effect at telomeres:**
   • *RAT1-TAP* construct was used to partially purify Rat1p for use in *in vitro* exonuclease experiments. These worked well and have shown that Rat1p can degrade both double stranded and single stranded DNA substrates, as detected by agarose gel electrophoresis and ethidium bromide staining. The results also suggest that Rat1p may act as a recombinase, as has been shown previously for a related protein, Sep1p. I've developed an i*n vitro* exonuclease assay that uses double stranded plasmid DNA as a substrate and detection of overhang production by in-gel hybridization. I've constructed a strain that expresses an exonuclease dead allele of *RAT1-TAP*, which will provide a crucial negative control.
   • Chromatin Immunoprecipitation (ChIP) assay: Using the RAT1-TAP allele I've been able to show that Rat1p co-purifies with telomeric chromatin. In addition, the amount of co-purifying telomere DNA increases as synchronized cultures progress through S-phase. However, the signal is weak and this assay still requires optimization.
   • G-tail assays: This assay involves the hybridization of telomere probes to non-denatured genomic DNA to detect the single stranded overhangs at telomeres. The resulting G-tail signal is then normalized to the signal that the same probe yields when hybridized to denatured genomic DNA. I've assayed the cell's ability to form G-tails after depletion of Rat1 in the *rat1-td* strain, a temperature-sensitive degron strain. After 6 hr in non-permissive conditions, the G-tail signal is dramatically reduced, indicating that Rat1p is required for resection at telomeres. This hypothesis has also been tested by overexpressing the exo-dead allele, rat1-D233A, in a cdc13-1 mutant. This strain ordinarily has an increased G-tail signal, which is reduced when the catalytically inactive Rat1p is overexpressed.
   • Mass spectrometric identification of proteins that co-purify with Rat1-TAP: this experiment has only been done once, but yielded very satisfactory results. Some of the more interesting hits included Est2p (catalytic subunit of telomerase), Stm1p (a protein that interacts physically and genetically with Cdc13p, as well as with ribosome biogenesis machinery, and ChIPs to telomeres, ARS, and rDNA), and Ctf18p (a subunit of an alternative RFC complex implicated in telomere maintenance). In general, the data suggest that there is a strong relationship between RNA processing proteins and telomere maintenance. Also, I was glad to note that none of the proteins that were identified are exonucleases, which means the *in vitro* activity I've observed is most likely due to Rat1p and not some other co-purifying enzyme.

• A PCR assay to detect telomere-telomere fusions (T-TFs) in *rat1* mutants: This assay is still being optimized, but preliminary data suggest that accumulate in *rat1-td* but not *rat1-td lig4* Δ, as expected.

2. **Experiments with dhp1-1 temperature sensitive mutant (s. pombe *RAT1*):**
•Southern blots probing for telomeres have detected the fusions that are predicted to arise when cells attempt to divide in the absence of dhp1. These experiments provide molecular evidence to compliment experiments in which these chromosome fusions are visualized *in vivo* by DAPI staining.
•A PCR assay has been used to detect circular chromosomes in the dhp1-1 mutant grown at restrictive temperature. I still need to clone the PCR products and verify them by DNA sequencing.

3. **Experiments to detect Rat1p at DSBs: currently being done**
• Do *rat1* mutants show a viability defect after induction of a DSB at the HO site? This question will be asked using the system developed in the Haber lab to monitor the processing and repair of a DSB during mating type switching. Cell viability will be measured simply as the ability to form colonies after a pulse of HO expression. The level of repair by HR can be determined by mating type tests.
• Using the same system, resection at the cut HO site will be monitored using an in-gel hybridization assay like the one used to assay G-tail formation.
• Experiments to test the role of Rat1p in repair of naturally forming DSBs during meiotic recombination will be done in the SK1 strain background. This strain sporulates to 100% efficiency in 12 hr, and is used when synchronous meiotic cultures are needed. The assays themselves will be identical to the ones used to monitor breaks at the HO site, except the breaks will be created at hotspots of meiotic recombination.

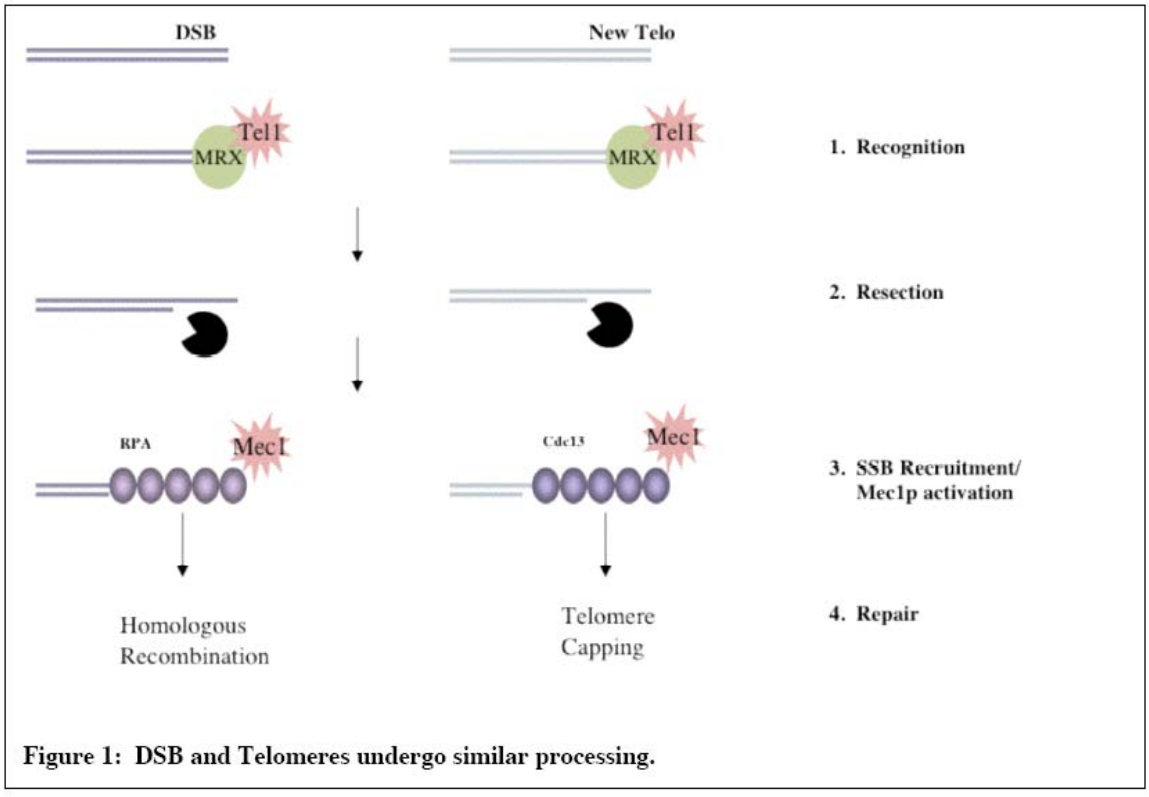

Figure 1: DSB and Telomeres undergo similar processing.

Key Research Accomplishments

- Used *in vitro* biochemical assays to show that Rat1p has the appropriate enzymatic activity to perform the telomere resection that creates G-tails.
- Used ChIP assays to show that Rat1p is present at telomeric regions and mass spectrometry to show that Rat1p co-purifies with known telomere-binding proteins.
- Determined that telomere end resection is impaired in cells that are depleted of Rat1p.
- Used PCR-based assays and Southern blotting to show that telomeres undergo end-to-end fusions when Rat1p is depleted or mutated, in both budding and fission yeasts.

Reportable Outcomes

- None yet.

Conclusions/Discussion

The results reported here provide compelling support for the hypothesis that the exoribonulcease, Rat1p, is the enzyme that resects telomeres at the end of S-phase. Although the enzyme responsible for this activity has never before been identified, much has been determined concerning its regulation and many proteins have been shown to be important for the efficient regulation of G-tail formation. Therefore, it will be interesting to see if Rat1p activity or its localization at telomeres is affected by the same proteins and activities that are known to regulate G-tail formation. In addition, determining whether Rat1p performs the same function in the repair of DSB by the HR pathway will be necessary to strengthen the parallels between telomere formation and DNA repair. Finally, *RAT1* is a highly conserved gene and experiments that look at the role of human *RAT1* in telomere processing and DNA repair should be done, as this activity is predicted to greatly influence genome stability and will likely have implications in the development of cancer.